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(54) Title: IMPROVED PHASE ANGLE AND MODULATION OF FLUORESCENT ASSAY

(57) Abstract

The presence or concentration of analytes in a sample can be assayed by observing with fluorescent lifetime techniques such as phase angle shift and modulation change the combined response to excitation of a fluorescent probe in the presence of the sample and a fluorescent material, the probe having a fluorescence intensity response to the presence or concentration of the analyte, the probe and the material having no substantial fluorescence lifetime change in response to the presence or concentration of the analyte and the material having a fluorescence lifetime in response to excitation that is different from the fluorescence lifetime of the probe.

Title

5   IMPROVED PHASE ANGLE AND MODULATION OF FLUORESCENT ASSAY

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Description

5   At present, there is considerable interest and
research activity in the field of chemical sensing.
Rapid and continuous monitoring of many analytes (pH,
pCO_2, O_2, metal ions, etc.) Is required in many areas of
science, including analytical chemistry, biochemistry,
10 environmental sensing, clinical chemistry and industrial
applications. Fluorescence-based sensing is one of the
promising   techniques   because   of   fluorescence
sensitivity, providing number of sensitive and specific
fluorescent probes for a variety of analytes and their
15 fabrication with fiber optics.

    At present, most types of commercial fluorescence
sensing devices are based on the standard intensity-
based   methods,   in   which   the   intensity   of   the
fluorescence produced by the probe molecule changes in
20 response to the analyte of interest. These intensity
changes can be induced by an analyte due to changes in
extinction   coefficient,   changes   in   quantum   yield,
absorption and emission spectral shifts, or simply due
to the   inner   filter   effects.   While   intensity
25 measurements are simple and accurate in the laboratory,
they are often inadequate in real-world situations. This
is because the sample may be turbid, the optical
surfaces may be imprecise and become dirty and optical
alignment may vary from sample to sample. A significant
30 disadvantage of intensity based sensing is the problem
of   referencing   the   intensity   measurements.   The

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fluorescence intensity measurement depends on the intensity of exciting light, the optical density at the excitation and emission wavelengths, the light loses in the optical path length, detector sensitivity and the
5   concentration of the fluorophore. These difficulties with intensity-based sensing appear to be limiting the more widespread use of fluorescence for quantitative chemical sensing.

Recent advances in optoelectronic have now made
10   possible a new type of fluorescence sensing. Instead of fluorescence intensities it is possible to measure fluorescence lifetime, particularly by the phase modulation method with a simple light sources. The advantages of lifetime-based sensing and the for a
15   several mechanisms of analyte-induced changes in lifetime are reviewed elsewhere in detail (Szmacinski and Lakowicz, Topics in Fluorescence Spectroscopy, Vol 4, pp.295-334, Plenum Press, New York 1994). The preferred lifetime-based sensing technique is phase-
20   modulation, where analyte-induced changes in lifetime of the probe are measured by phase angle and modulation at single modulation frequency. The phase and modulation are related to the analyte concentration. A number of lifetime-sensitive probes have been characterized for
25   several analytes such as pH, Ca^{2+}, Mg^{2+}, K^+, Na^+. Practically all of the known analyte lifetime-sensitive probes excluding the probes for O_2 sensing display short lifetimes, most often in the range of 1- 5 ns. Using probes with short lifetimes requires high modulation
30   frequencies in the range of 50 - 300 MHz in order to obtain sufficient changes in phase and modulation for

analyte sensing. However, even though inexpensive light sources such as LED's can be modulated in that range of frequencies, the cost of phase modulation device is still sufficiently expensive to inhibit broad commercial use.

There is observed a significant effort in several laboratories to develop functional long lifetime probes having a fluorescence lifetime in the range above 100 ns. There is a large number of fluorophores that display long lifetime fluorescence, such as metal-ligand complexes based on ruthenium, rhenium, osmium, platinum or rhodium. Lifetimes as long as 100 µs can be obtained using such probes. However, there are not known such metal-ligand complex-based probes that are sufficiently sensitive to analytes for practical use. Only O_2 probes based on metal-ligand complexes in which the mechanism of quenching is exploited are presently used widely. The advantage of longer lifetime probes is that sensing can be at a relatively lower modulation frequency, for example, in the range of 10-1000 kHz. A phase and/or modulation instrument based on the use of longer lifetime fluorescent materials, such as the metal-ligand complex based materials can be designed to use inexpensive components due mainly to the lower frequencies required.

It is among the objects of the present invention to overcome the disadvantages of the present technology.

It is one object to avoid the need for development and manufacture of relatively exotic long lifetime fluorescent materials such as those based on metal-ligand complexes.

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It is another object of this invention to avoid the need for the use of high frequencies in phase-modulation assays, so that relatively less expensive lower frequency electronic components can be used.

5   It is yet another object of this invention is to measure phase and modulation in the low modulation frequencies using available (or designed) fluorophores in which the intensity is sensitive to the analyte of interest.

10   It is a further object of this invention to avoid the problems encountered in the past with intensity-based fluorescent assays due to ambient light, turbidity in the sample, the need for optical couplings, intensity dissipated in wave guides, and the like.

15   These and other objects are accomplished by the present invention, which makes use of the discovery that relatively low frequency phase and modulation techniques can be used to assay for analytes by employing, in combination, a fluorescent probe that shows a
20   fluorescent intensity response in the presence of the analyte but has a fluorescent lifetime that is substantially unaffected by the presence of the analyte and a fluorescent material having a fluorescent lifetime that is different from the fluorescent lifetime of the
25   probe, the fluorescent material being substantially unaffected in both fluorescence lifetime and fluorescence intensity by the presence of the analyte.

Although the mechanism by which this invention works is not fully understood at the present time, it is
30   believed that the phase angle and modulation of the sample depend on values of lifetime and fractional

intensities of components. The changes in phase angle and modulation can be as result of changes in fractional intensities without changes in the lifetime of both component. By mixing an analyte sensitive fluorophore 5 (short lifetime or long lifetime) with the second fluorophore that is not analyte sensitive (long lifetime or short lifetime) and by correctly selecting the relative concentration of the fluorophores, the excitation wavelength, and the emission band observed, 10 an analyte sensitive probe can be created. The expected analyte induced changes in phase angle and modulation can be as large as 90 degree and 1.0, respectively. To observe large changes in phase angle and modulation the modulation frequency can be at a lower range, determined 15 by the long lifetime component.

The controlled mixing of two fluorophores allows using any intensity-based fluorophore regardless of its lifetime as a lifetime-based probe using a phase and modulation technique. The analyte-induced changes in 20 fractional intensities of two components allow the determination of the analyte concentration from the phase and/or modulation at a single modulation frequency.

The properties, requirements, advantages and 25 various applications of the present invention are discussed below with reference to the drawings.

Brief Description of the Drawings

30   Figs. 1a and Fig. 1b show the expected frequency responses of phase angle and modulation for long

lifetime and short lifetime fluorophores.

Fig. 2a and Fig 2b show the expected frequency responses of phase angle and modulation of fluorescence that consist of fraction of long lifetime and fraction
5   the short lifetime fluorescence.

Fig. 3a and Fig. 3b show the expected frequency responses of phase angle and modulation where the value of short lifetime is changed from 0.5 to 10 ns in several steps.

10   Fig. 4a and Fig 4b show the expected frequency responses of phase angle and modulation where the value of long lifetime is 100, 500, and 5000 ns and short lifetime fluorescence of 10 ns.

Figs. 5a, 5b, 6 and 7 illustrate Example 1.
15   Figs. 8, 9 and 10 illustrate Example 2.
Figs. 11, 12, 13, 14 and 15 illustrate Example 3.

Detailed Description

Referring again to Figs. 1a and 1b, two distinct
20   ranges of modulation frequencies are needed to measure the short lifetime and to measure long lifetime fluorescence. In order to measure the lifetimes shorter than 5 ns that display most of organic fluorophores, high modulation frequencies are required in order of 100
25   MHz. This is normally achieved by an expensive phase-modulation fluorometers which are commonly used in research labs. Long lifetime fluorescence requires low modulation frequencies in the range of 100 kHz. The design of a phase-modulation instrument for low
30   modulation frequencies is less expensive and can provide higher accuracy of measurements that similar devices

with high frequency. However, there are not presently widely available lifetime-sensitive fluorescence sensors that display long lifetimes other than those employing metal-ligand complexes and used in oxygen sensing as
5 described above.

Figs. 2a and Fig 2b show the expected frequency responses of phase angle and modulation of fluorescence that consist of a fraction of long lifetime and a fraction the short lifetime fluorescence. The values
10 from 0 to 1 represent the fractional intensity of short lifetime fluorescence in the measured signal. There are observed great changes from 0 to about 90 degrees in phase angle and from 1 to 0 in modulation values upon changes of fractional contribution of fluorescence from
15 both fluorophores. In addition the steeples part of the modulation value is equal the fractional intensity of short lifetime fluorescence. Thus fractional-dependent phase angle and/or modulation can be used to measure the intensity of a desired fluorophore in the sample using
20 in most cases only one modulation frequency. The changes in fractional intensity can be induced by the analyte; (1) by affecting the absorption spectra (extinction coefficient and/or spectra shift), (2) by affecting the emission spectra (quantum yields and/or spectra shifts).
25 There are many possibilities to optimize such a sensor probe by the choice of excitation wavelength, emission band and relative concentration of used fluorophores.

Referring again to Figs. 3a and 3b, the fractional intensity of the short fluorescence lifetime component
30 in each case is the same for 0.15 in Fig. 3a and 0.5 in Fig.3b. The important observations from these figures

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are that the phase angle and modulation below certain frequency are not sensitive to the value of short lifetime fluorescence in the sample. This is similar to the gating technique in the pulse method where, by
5   applying a certain delay after pulse excitation, only the signal from long lifetime fluorescence is detected. In the phase-modulation technique it is impossible to measure only long the lifetime component. Analytical methods have been developed for background correction in
10 phase-modulation fluorometry based on the measurements of the background sample or based on known intensity decay of background and it contribution in the sample signal. In the case presented in Fig. 3a and 3b the desired intensity of the long lifetime component can be
15 obtained by measuring the phase and/or modulation at single modulation frequency regardless of intensity decay of the background or autofluorescence or scattered light until the mean lifetime is short enough compared to a long lifetime fluorophore. This feature can be used
20 also to determine the anisotropy of the long lifetime component in turbid media with scattered light or with background fluorescence having a known value of anisotropy. This may find immediate application in detecting binding of high molecular weight
25 macromolecules labeled with a metal-ligand complexes or in immunoassays. It is also important for chemical sensing that changes in the lifetime due to presence of an analyte for short lifetime indicators have no effect of fractional intensity and thus on sensing of analyte
30 concentration.

Referring again to Figs 4a and 4b, the most

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important observation is from Fig 4b where the value of
steeples part of modulation reflect the fractional
intensity of the short lifetime component regardless on
the value of the long lifetime component. Also, it is
5   important that the choice of low modulation frequency
depends mostly on the value of the long lifetime
component but not on the value of short lifetime
component.

Examples

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The present invention is further illustrated with
reference to the following examples. Example 1
demonstrates the phase and modulation sensitivity when
the fractional intensity of sample is varied by various
15 relative concentrations of two dyes in the sample.

Example 2 demonstrates the possibility to determine
the intensity of flurophore of interest in presence of
various amoun of background or autofluorescence from the
solvent.

20   Example 3 demonstrate how the sensing probe can be
created when pH induced changes in fractional
intensities of a probe contained pH intensity sensitive
indicator and long lifetime fluorophore con be measured
by phase angle and modulation.

25

Example 1

Two fluorophores have been chosen, one with a long
lifetime fluorescence from metal-ligand complexes like
[Ru(bpy)$_2$dcbpy]^2 with a lifetime in glycerol of 1060 ns
30 and the second with short lifetime like many organic
fluorophores Texas Red Hydrazide with a lifetime of 3.4

ns in glycerol. The two dyes were mixed at various relative concentrations to induce the various fractional intensities in the sample

Fig. 5a show the absorption spectra of long
5   lifetime fluorophore [Ru(bpy)$_2$dcbpy]$^{2+}$ and short lifetime fluorophore Texas Red Hydrazide (TRH) (solid lines and their mixture at concentrations specified in Figure. It is shown that any excitation wavelength shorter than about 640 nm will excite both fluorophores. The
10   resulting fractional intensities from both fluorophores will be strongly dependent on the choice of excitation wavelength. One can imagine that value of extinction coefficient or shift in absorption spectrum will result in changes of fractional intensities that can be
15   monitored with phase and/or modulation measurements. One excitation wavelength has been chosen as 488 nm (Argon-ion laser). The total concentration of dyes were low to avoid the inner filter effects. The changes in absorption was induced by using various concentration
20   combination of both fluorophores.

Fig. 5b shows the emission spectra of [Ru(bpy)$_2$dcbpy]$^{2+}$ and TRH at one selected concentration combination. The emission spectra overlap well and for phase and modulation measurements we used the long pass
25   filter above 550 nm.

Fig. 6 show the frequency responses of phase angle for long lifetime fluorophore [Ru(bpy)$_2$dcbpy]$^{2+}$ with a lifetime of 1060 ns and the short lifetime TRH of 3.4 ns when mixed together at a specified relative
30   concentrations from 0 to 12.8. The obtained values for fractional intensities are in good agreement with those

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expected from steady-state measurements of full emission spectra. These experimental data are related to those simulated and discussed in Fig. 2a.

Fig.7 show the frequency responses of modulation for long lifetime fluorophore $[Ru(bpy)_2dcbpy]^{2+}$ with a lifetime of 1060 ns and the short lifetime TRH of 3.4 ns when mixed together at a specified relative concentrations from 0 to 12.8. These experimental data confirm that presented and discussed in Fig. 2b.

Example 2

The purpose of this example was to demonstrate the calculation of intensity of long lifetime fluorophore in presence of background fluorescence from the solvent. Long lifetime fluorophore was the same as in Example1 $[Ru(bpy)_2dcbpy]^2$ with a lifetime in glycerol of 1060 ns. The glycerol (from Fluka) displayed a background fluorescence that overlaps with the emission of ruthenium. In many applications the requirements are for very low dye concentration which posses the difficulties for increased background corrections. The increased contribution of background fluorescence from solvent was obtained by the dilutions of the ruthenium sample with glycerol.

Fig. 8 show the emission spectra of ruthenium with decreased concentrations and also background fluorescence from used glycerol. The fractional intensity of glycerol calculated by integrating the spectra are following: 0.108, 0.379, 0.757, and 0.886 at ruthenium concentration of 740, 150, 29 and 6 nM.

Fig. 9 show frequency responses of phase angle of

the samples with increased contributions of background
fluorescence. The obtained values are in good agreement
with those from steady-state measurements. The small
difference are because of different excitation sources

5   (xenon lamp and monochromator in steady-state, and Ar-
ion laser in phase-modulation measurements). It should
be noted that phase angle is related only to fractional
intensity at modulation frequencies lower than 1 MHz.
The glycerol displayed a complex intensity decay with a

10   mean lifetime shorter than 3.5 ns. These experimental
data confirm that presented in Fig 3a where the short
lifetime component do not contribute to changes in phase
angle for certain low modulation frequencies.

    Fig 10   show frequency responses of modulation of

15   the samples with increased contributions of background
fluorescence. The steeples part of modulation indicate
good separation between the fluorescence of ruthenium
and that of glycerol and can be easy used to determine
the absolute intensity of the ruthenium. These results

20   confirm that discussed in Fig. 3b.

    Example 3
        The goal of this example is to demonstrate the
    great opportunity of designing the fluorescence probe

25   for measuring a large variety of chemical species where
    the change in fluorescence intensity can be obtained.
    For example we have chosen a pH intensity sensitive
    indicator Naphtofluorescein and the second dye with a
    long lifetime $[Ru(phn)_3]^{2+}$. The Naphtofluorescein as most

30   of fluorescein dyes display pH sensitive absorption
    spectrum and decreased fluorescence quantum yield at

lower values of pH. To demonstrate the practical use of such sensor we used inexpensive blue LED as a excitation source.

Fig. 11 shows the emission spectra of a mixture of ruthenium and Naphtofluorescein at various values of pH. The increased pH values affect the fractional intensities from both of dyes which is displayed as decreased fluorescence from the ruthenium and increased contribution from the Naphtofluorescein. The fractional intensities in the sample can be selected by the cutt off filter or by band pass filter. We have chosen use long pass filter above the 595 nm. The excitation source was a blue LED with a maximum intensity at 475 nm.

Fig. 12 shows the frequency responses of phase angle of such pH sensor. There are observed remarkably large changes in phase angle at modulation induced by the pH of a sample. The pH phase-based sensing can be performed at low modulation frequencies in spite of very short lifetime of Naphtofluorescein of about 0.45 ns frequencies below 10 MHz.

Fig. 13 shows large changes in modulation induced by pH of the sample. There is a wide range of modulation frequencies where modulation value is related only to the pH value even not to modulation frequency. This is because the difference in lifetimes of ruthenium and Naphtofluorescein is very large about 1000-fold. It is again important to note that long lifetime value determines the useful low modulation frequency for sensing.

Fig. 14 shows pH-dependent phase angle for several modulation frequencies. It should be noted the magnitude

of phase angle changes up to 69 deg (see values in the brackets) . This is remarkably pH sensor, which allows measurements the pH changes as small as of 0.0035 of pH unit assuming that phase angle can be measured with an

5   accuracy of 0.1 deg (from curve at 2200 kHz in the range from pH 6 to 8) . Also choosing the modulation frequency allow to shift the apparent pKa, in presented case from 6.41 to 7.24.

Fig. 15 shows pH-dependent modulations for several

10 modulation frequencies. The pH induced changes in modulation (values in the brackets) are large and significantly depends on the choice of modulation frequency. The apparent pKa is slightly dependent on modulation frequency.

15 The above description is intended to be illustrative of the present invention, which is intended to be limited only by the claims that follow.

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Claims

10   What is claimed is:

1.   A method for performing an assay for the presence
      or concentration of an analyte in a sample, the
15   method comprising:

      a.   contacting the sample with a fluorescent probe
            that, when in the presence of the analyte and
            illuminated with activating radiation, emits
20   fluorescent radiation at an intensity that is
            related to the presence or concentration of
            the analyte, the fluorescent radiation having
            a first lifetime that is substantially
            unchanged by the presence or concentration of
25   the analyte in the sample;

      b.   illuminating with modulated activating
            radiation of one or more frequencies or
30   amplitudes, or both, the fluorescent probe and
            a fluorescent material that emits, in response

to said activating radiation, fluorescent
radiation having a second fluorescent lifetime
that is substantially unchanged by the
presence or concentration of the analyte and
5   that has an intensity that is substantially
unchanged by the presence or concentration of
the analyte and wherein the second fluorescent
lifetime is longer or shorter than the first
fluorescent lifetime;
10

c.   sensing change in phase angle or modulation,
or both, of the mixed emissions from the
fluorescent probe and fluorescent material
15   upon such contacting; and

d.   determining from such change the presence or
concentration of the analyte.
20

2.   The method of Claim 1 wherein the sample is
contacted by the fluorescent material.

25   3.   The method of Claim 1 wherein the sample is
contacted with a matrix material that supports the
fluorescent probe and, optionally, the fluorescent
material.

30

4.   The method of Claim 1 wherein the sample is mixed

with the fluorescent probe and, optionally, with the fluorescent material.

5. An assay device for use in determining the presence or concentration of an analyte in a sample, the device comprising

a. a fluorescent probe that, when in the presence of the analyte and illuminated with activating radiation, emits fluorescent radiation at an intensity that is related to the presence or concentration of the analyte, the fluorescent radiation having a first lifetime that is substantially unchanged by the presence or concentration of the analyte in the sample;

b. a fluorescent material that emits, in response to said activating radiation, fluorescent radiation having a second fluorescent lifetime that is substantially unchanged by the presence or concentration of the analyte and that has an intensity that is substantially unchanged by the presence or concentration of the analyte and wherein the second fluorescent lifetime is longer or shorter than the first fluorescent lifetime; and

c. a support member adapted for supporting the probe and, optionally, the material in contact with the sample during illumination of the probe and the

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material by the activating radiation.

6. The device of Claim 5 wherein the support member
   comprises a polymeric matrix.

7. An assay composition for use in assaying for the
   presence or concentration of an analyte in a
   sample, the composition comprising:

   a. a fluorescent probe that, when in the presence
      of the analyte and illuminated with activating
      radiation, emits fluorescent radiation at an
      intensity that is related to the presence or
      concentration of the analyte, the fluorescent
      radiation having a first lifetime that is
      substantially unchanged by the presence or
      concentration of the analyte in the sample; and

   b. a fluorescent material that emits, in response
      to said activating radiation, fluorescent
      radiation having a second fluorescent lifetime
      that is substantially unchanged by the presence
      or concentration of the analyte and that has an
      intensity that is substantially unchanged by the
      presence or concentration of the analyte and
      wherein the second fluorescent lifetime is
      longer or shorter than the first fluorescent

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lifetime.

8.   A kit for use in performing an assay for the
presence or concentration of an analyte, the kit
comprising:

a.   a container containing a fluorescent probe
that, when in the presence of the analyte and
illuminated with activating radiation, emits
fluorescent radiation at an intensity that is
related to the presence or concentration of
the analyte, the fluorescent radiation having
a first lifetime that is substantially
unchanged by the presence or concentration of
the analyte in the sample; and

b.   a container containing a fluorescent material
that emits, in response to said activating
radiation, fluorescent radiation having a
second fluorescent lifetime that is
substantially unchanged by the presence or
concentration of the analyte and that has an
intensity that is substantially unchanged by
the presence or concentration of the analyte
and wherein the second fluorescent lifetime is
longer or shorter than the first fluorescent
lifetime.

20
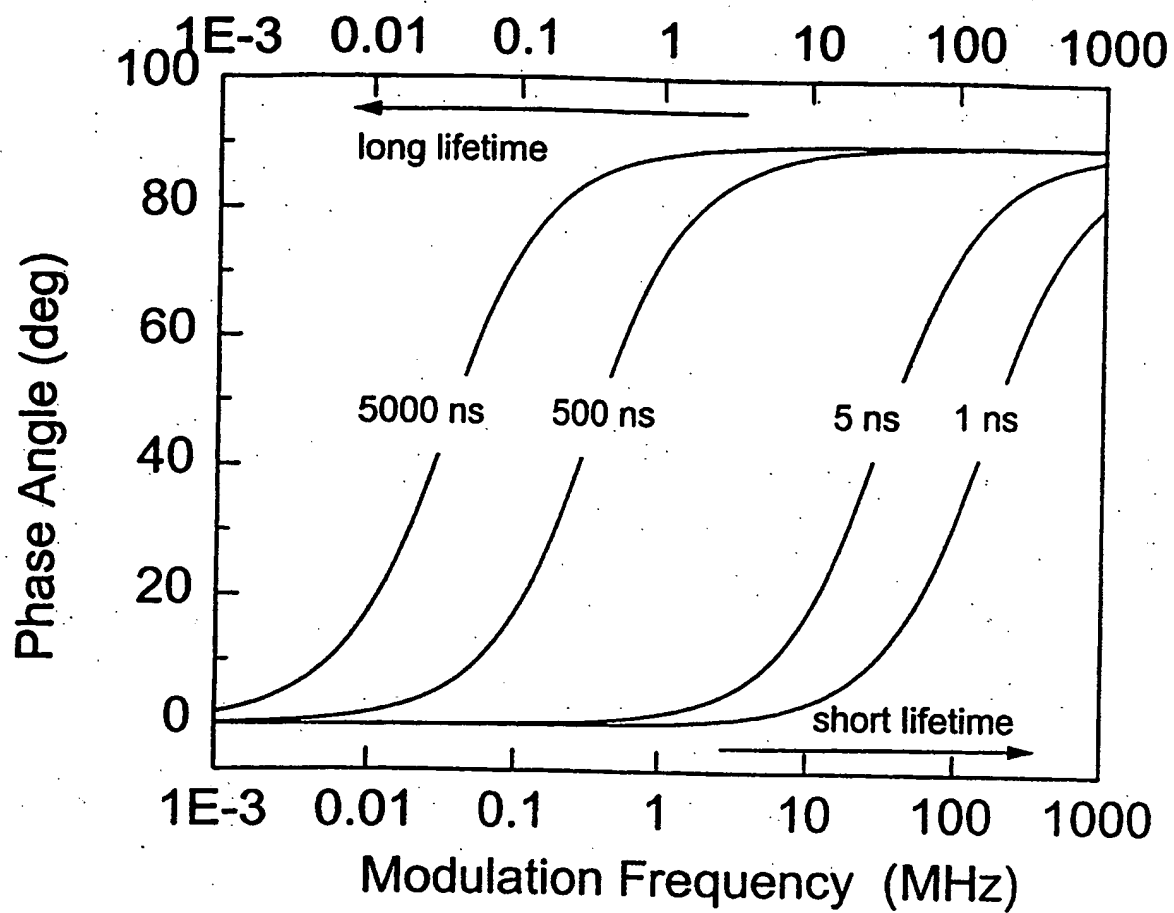
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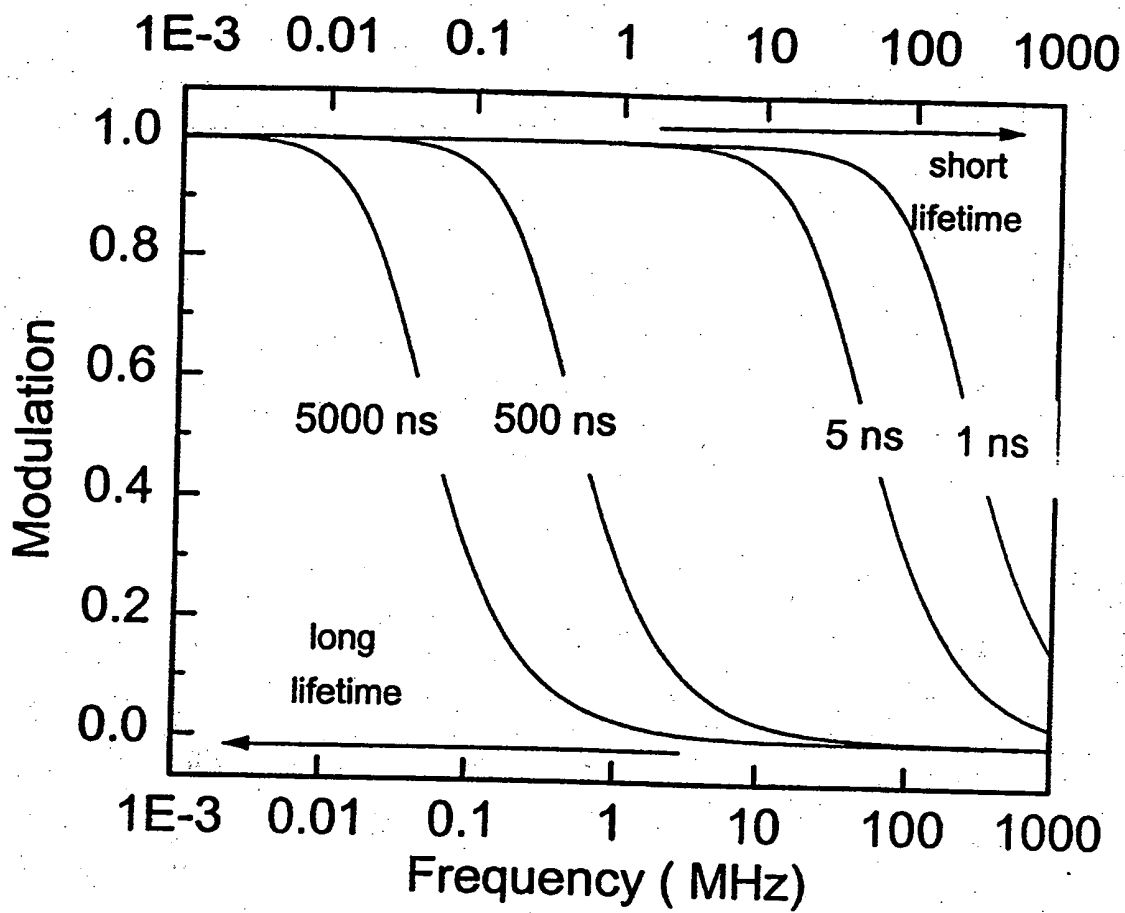
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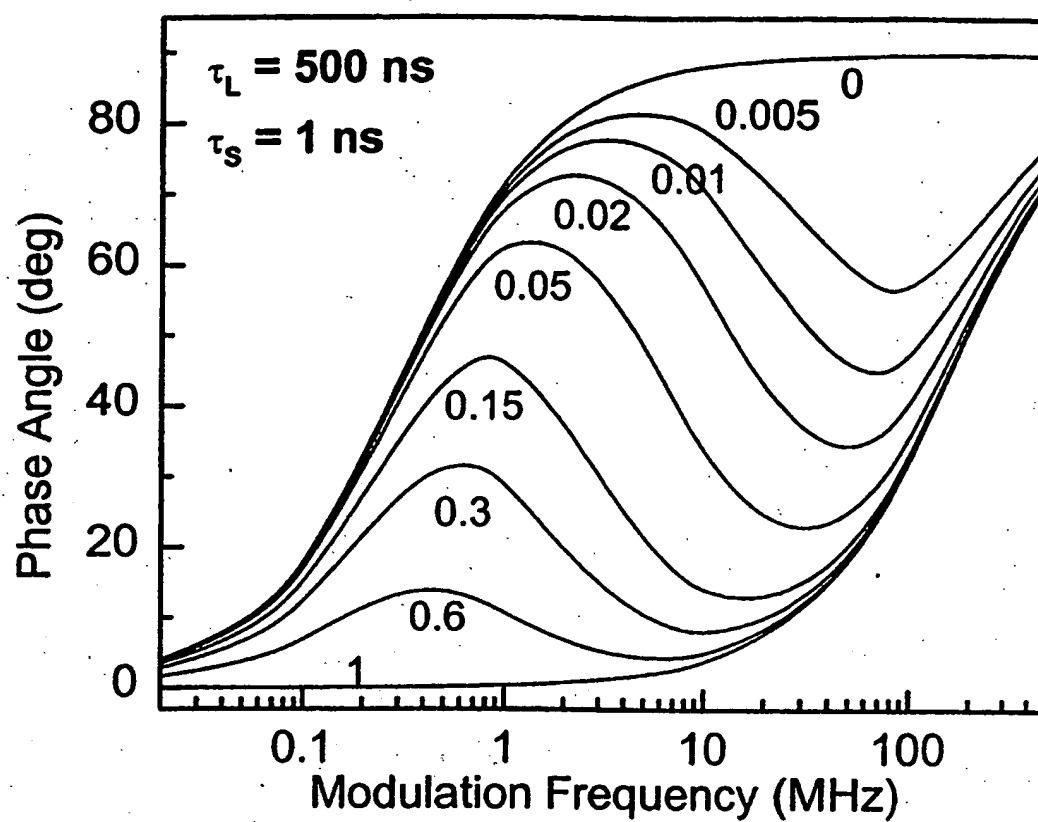
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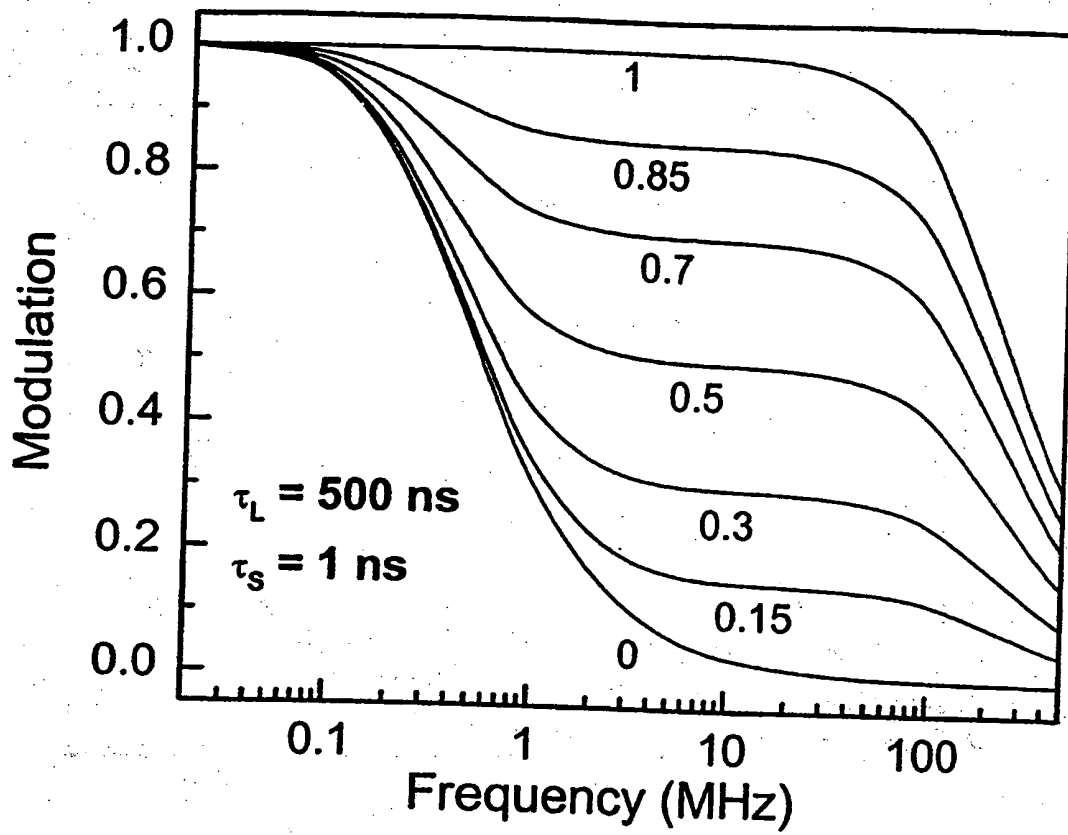
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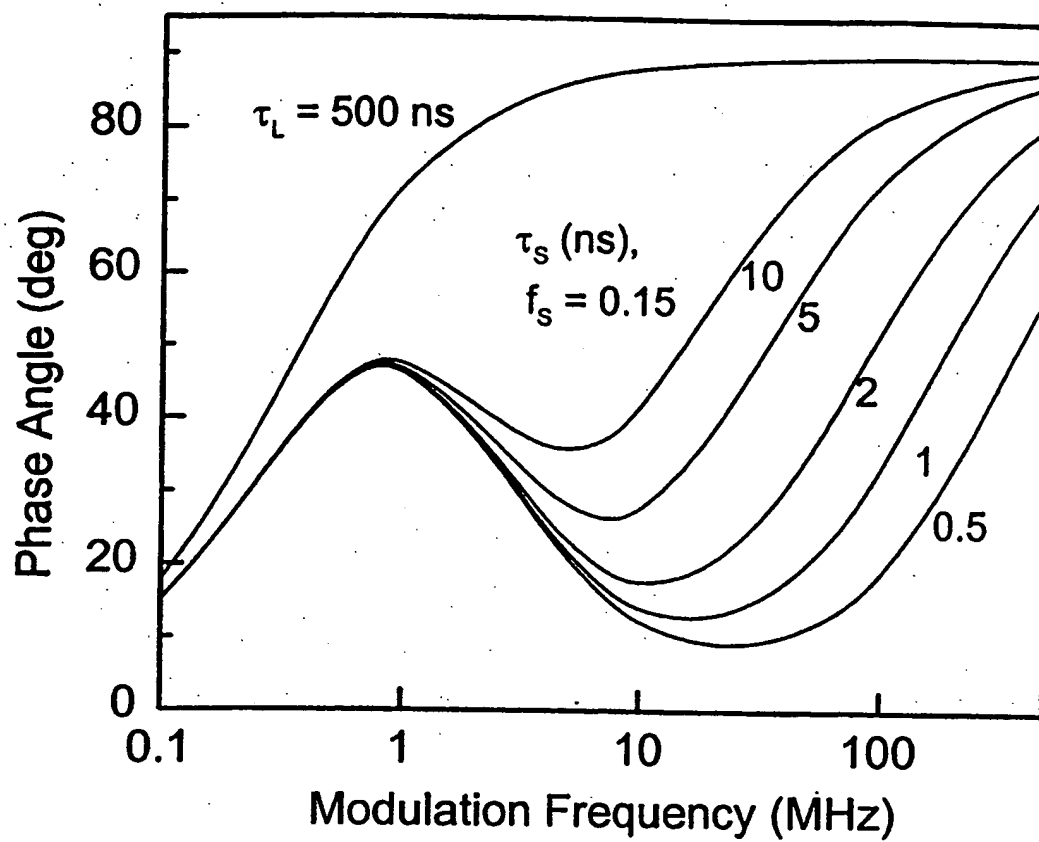
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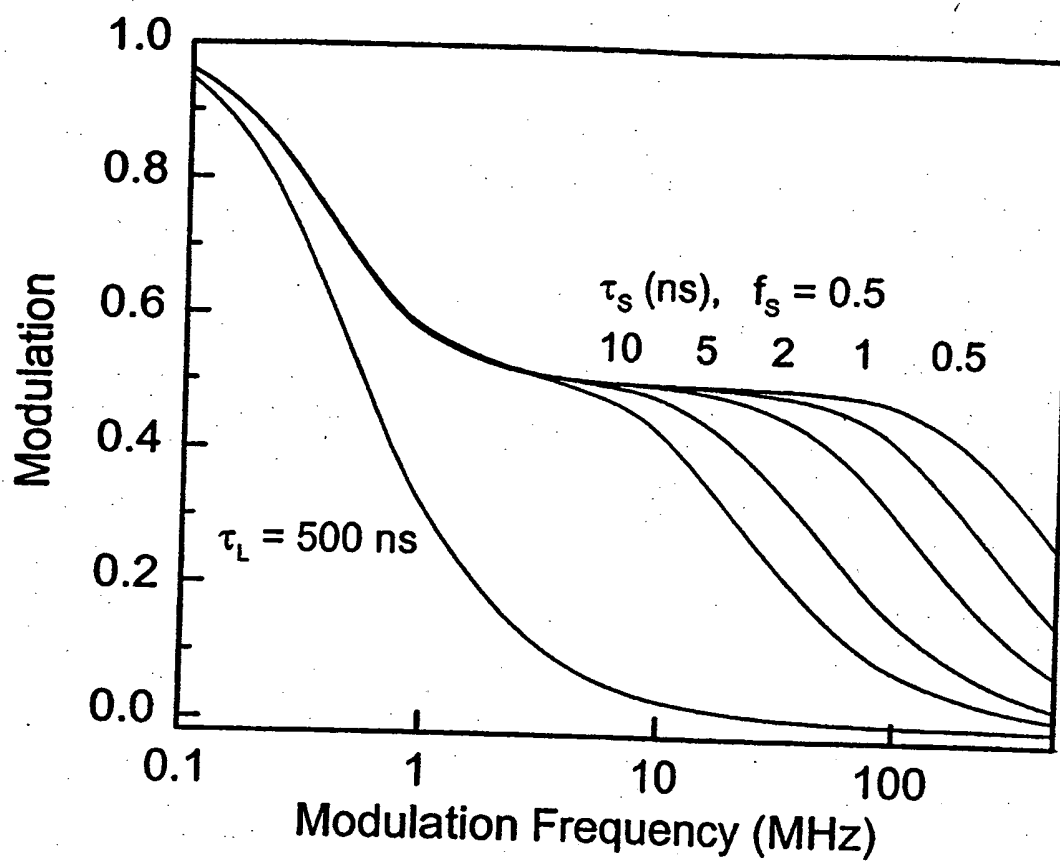
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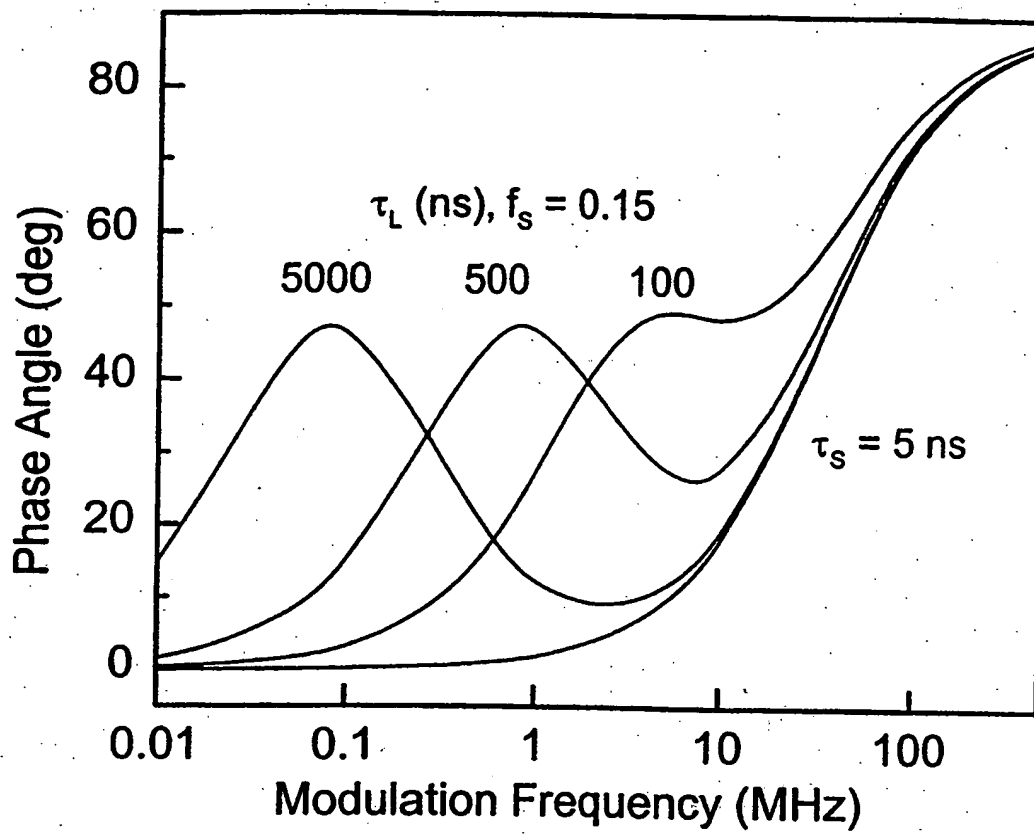
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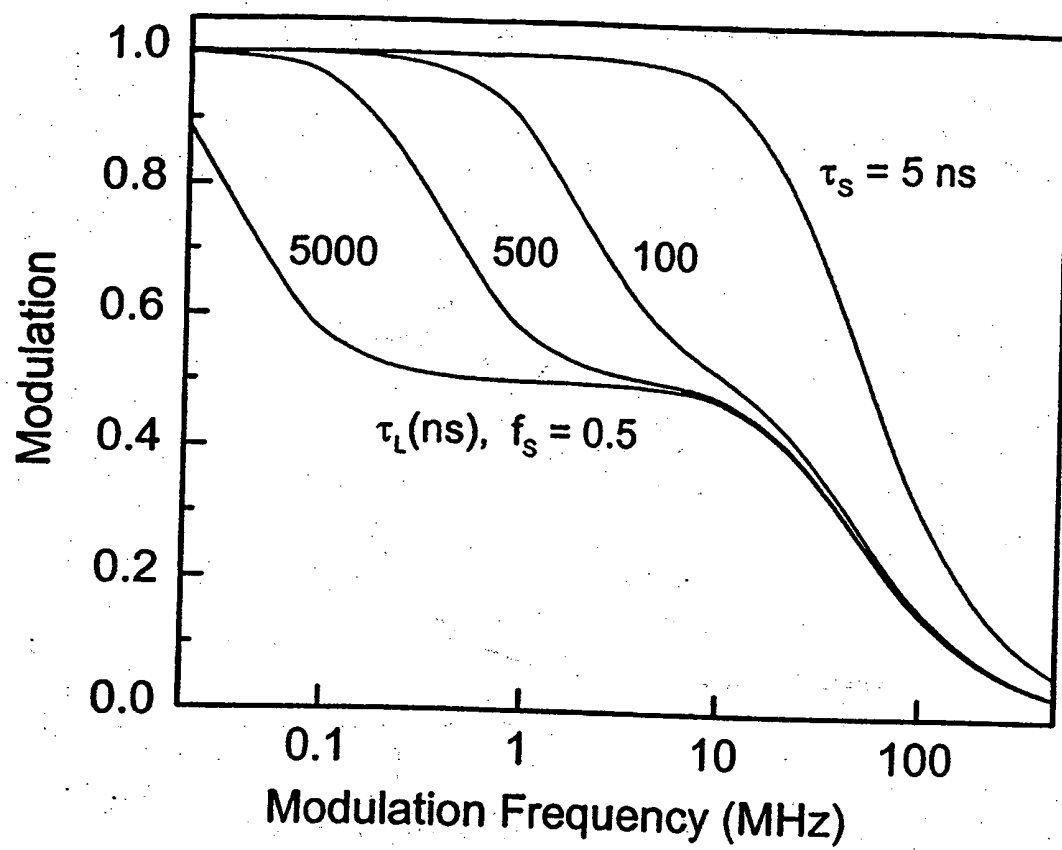
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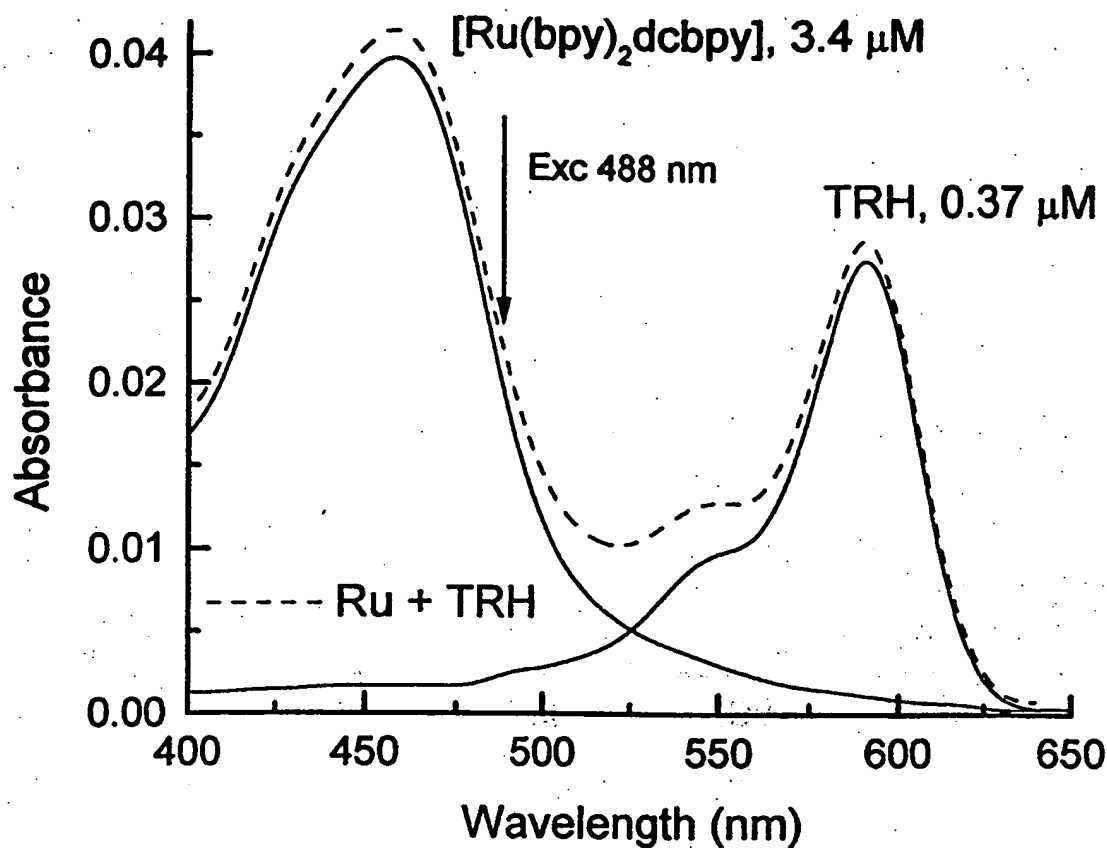
Serie B   OD Ru = 0.21   TRH = 0.0024 (488 nm)

Dyes were in glycerol (Fluka).

Concentration calculated base on extinction coefficients :

Ru - 12,000 $M^{-1}cm^{-1}$, [Evald]

TRH- 80,000 $M^{-1}cm^{-1}$ [MP]

52.

Ru + TRH (750 + 375 nM)
Exc 488 nm, in glycerol, t=22 C
TRH (375 nM)
Ru (750 nM)
Glyc.
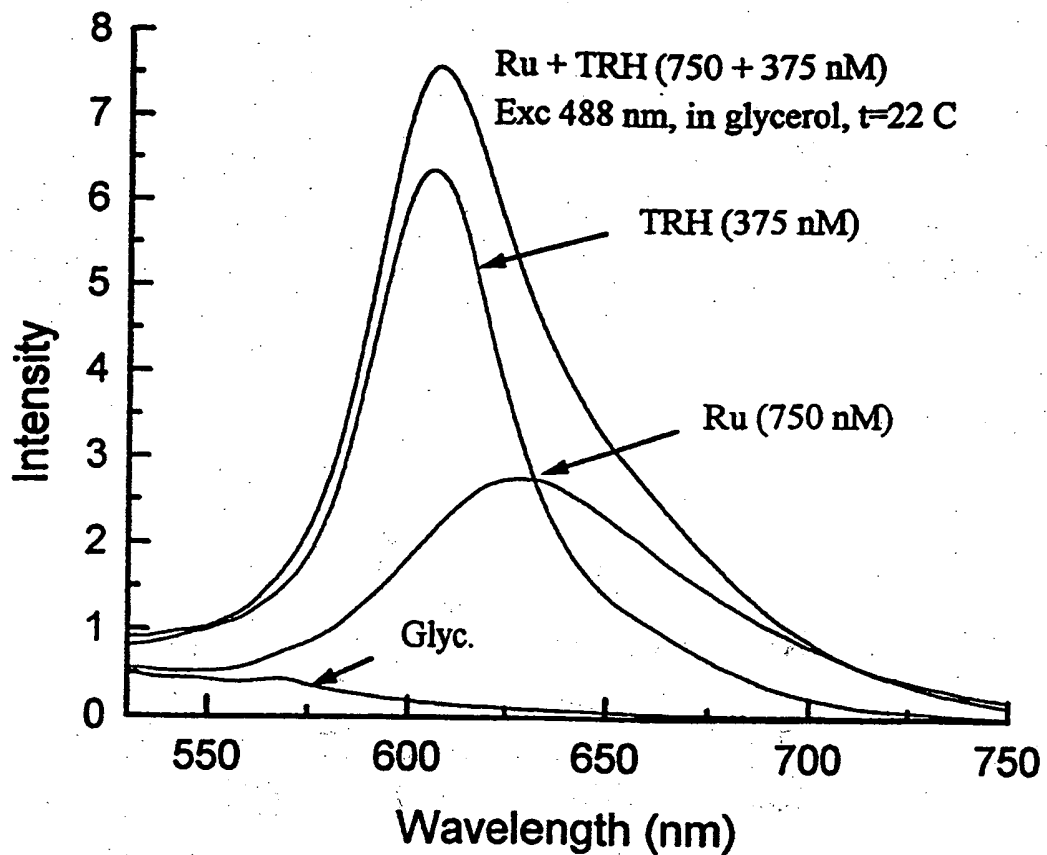
Fractional intensity in the mixture (serie C):

glyc. - 0.065

Ru - 0.439 $\}$ .417   OD = 0.0042

TRH - 0.613 $\rfloor$ .587   = 0.0024 $\left| \begin{array}{l} Int \ 1.41^{x} \\ OD \ 0.57^{x} \end{array} \right\} \ \phi = 2.4$

If separately it t is about 11% more than in mixture.

S t
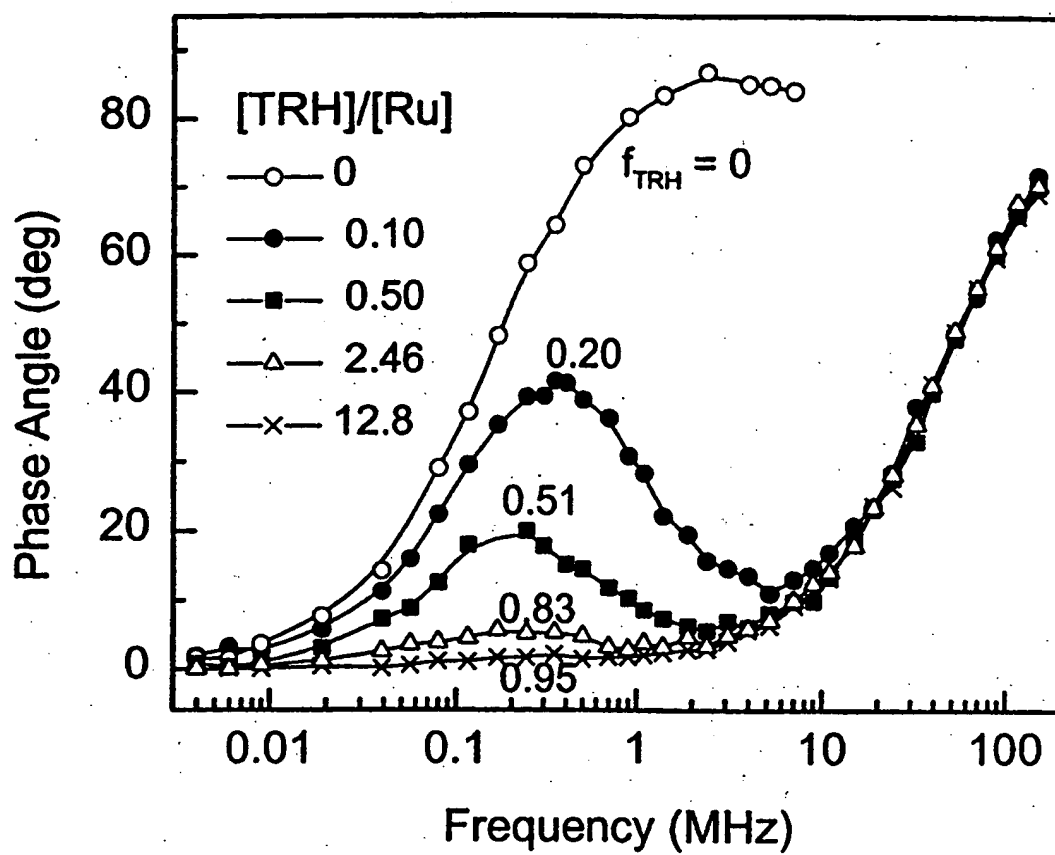
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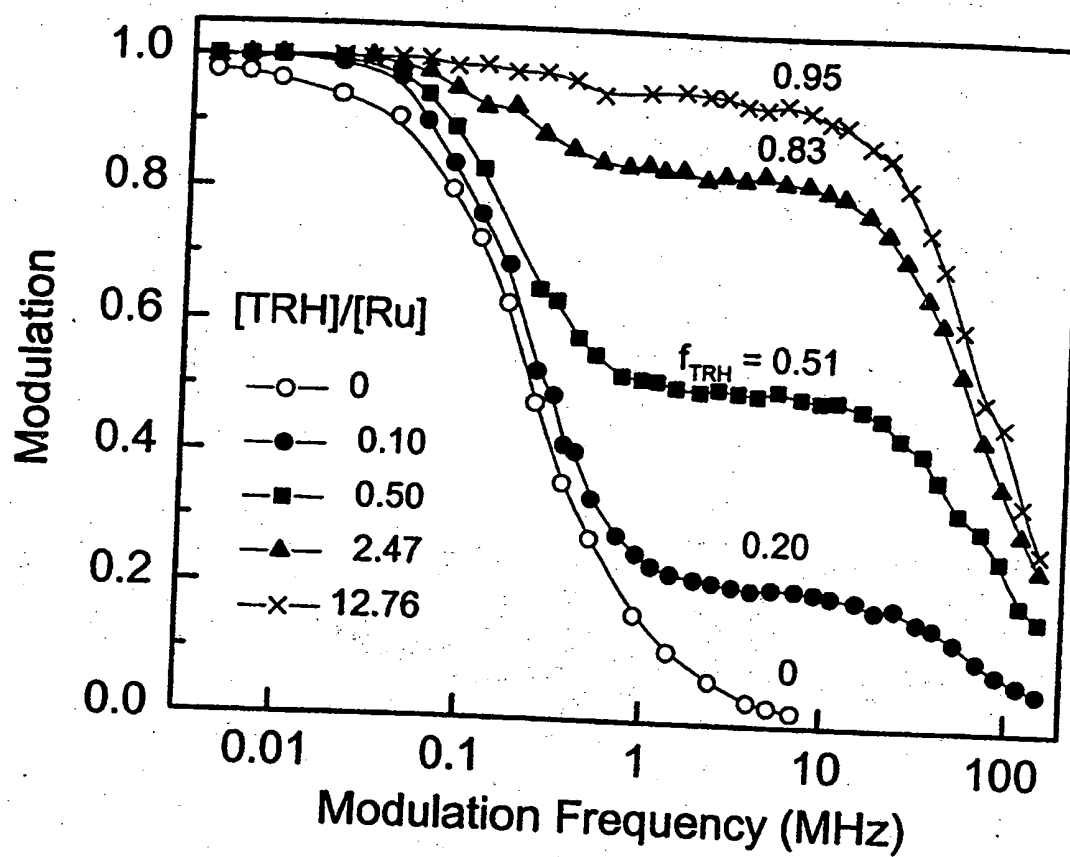
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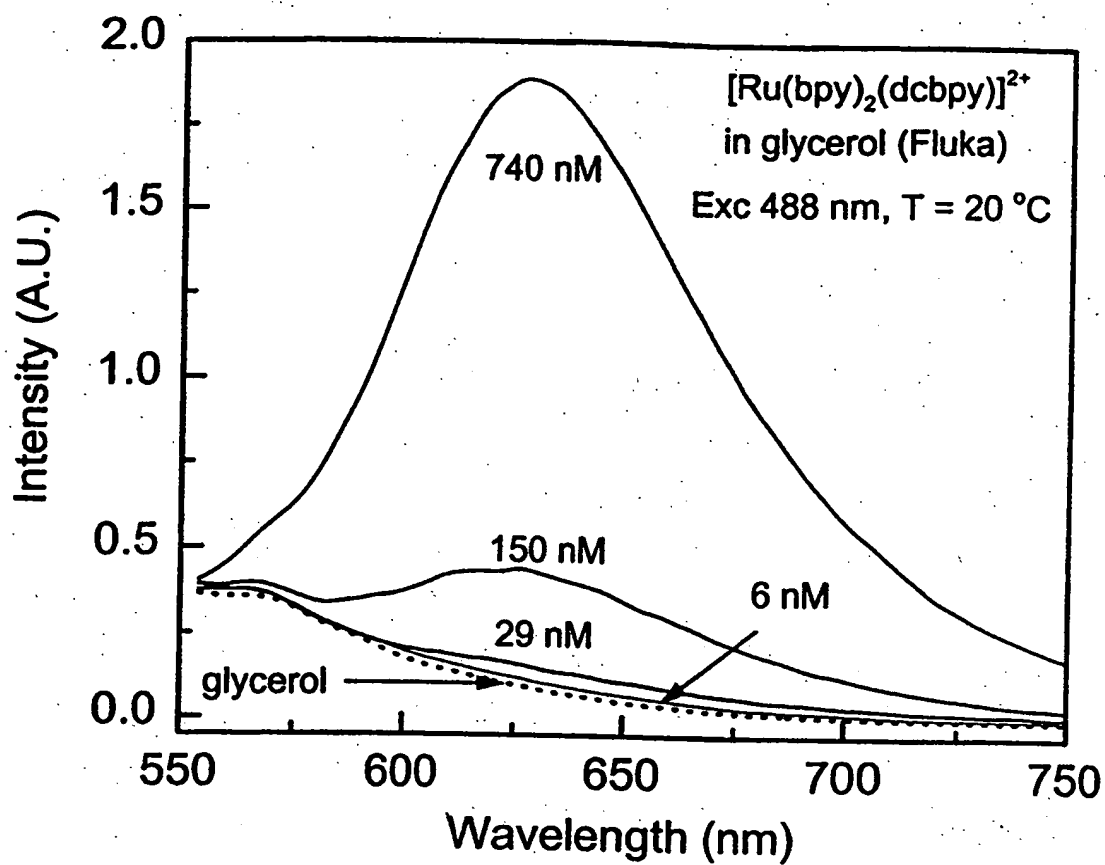
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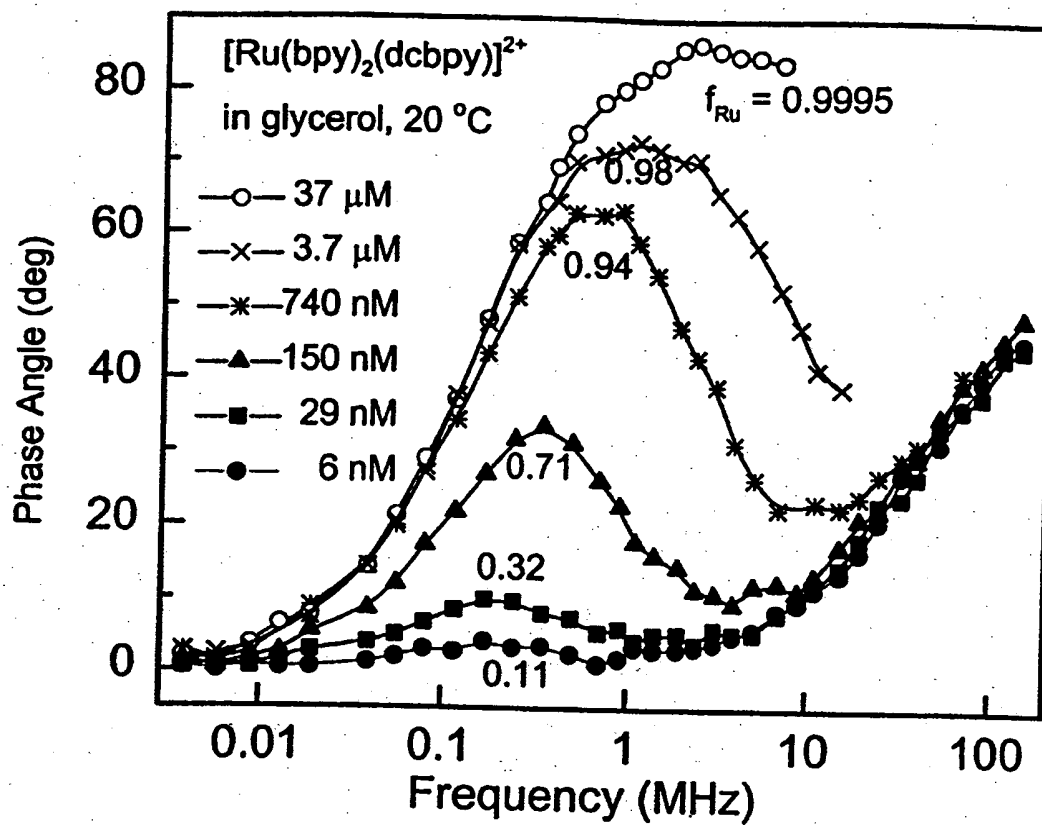
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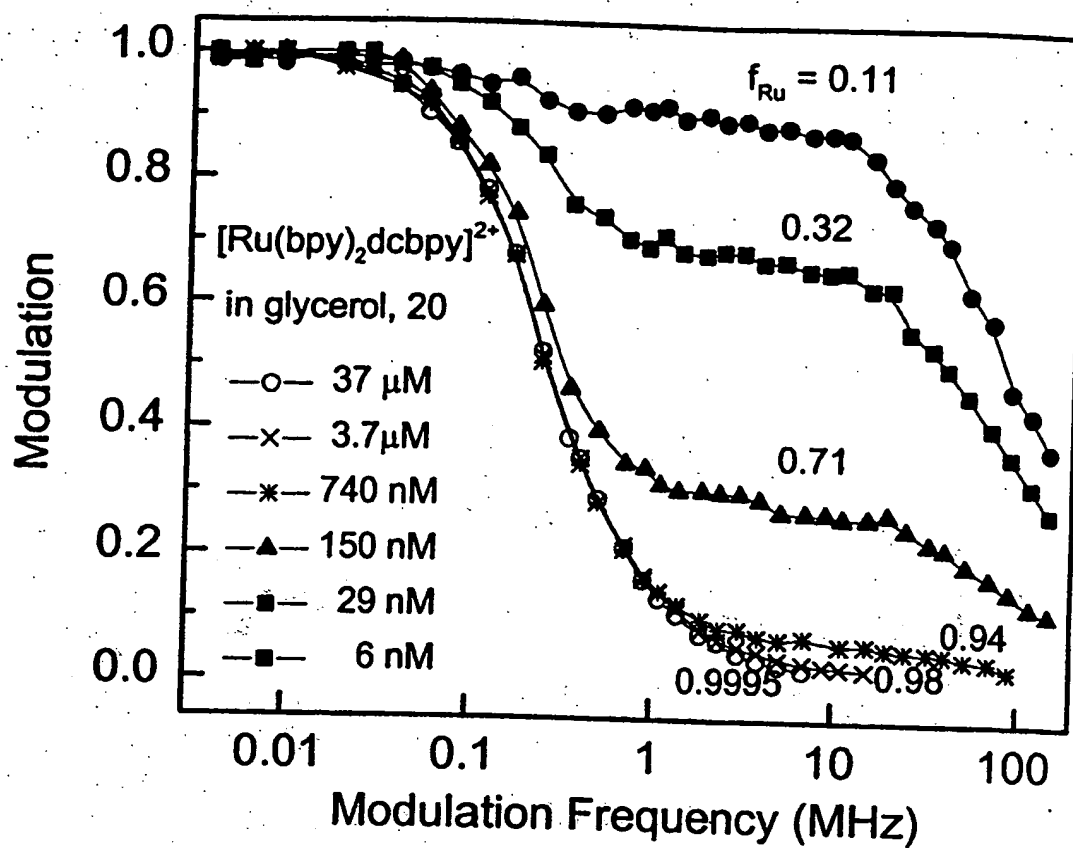
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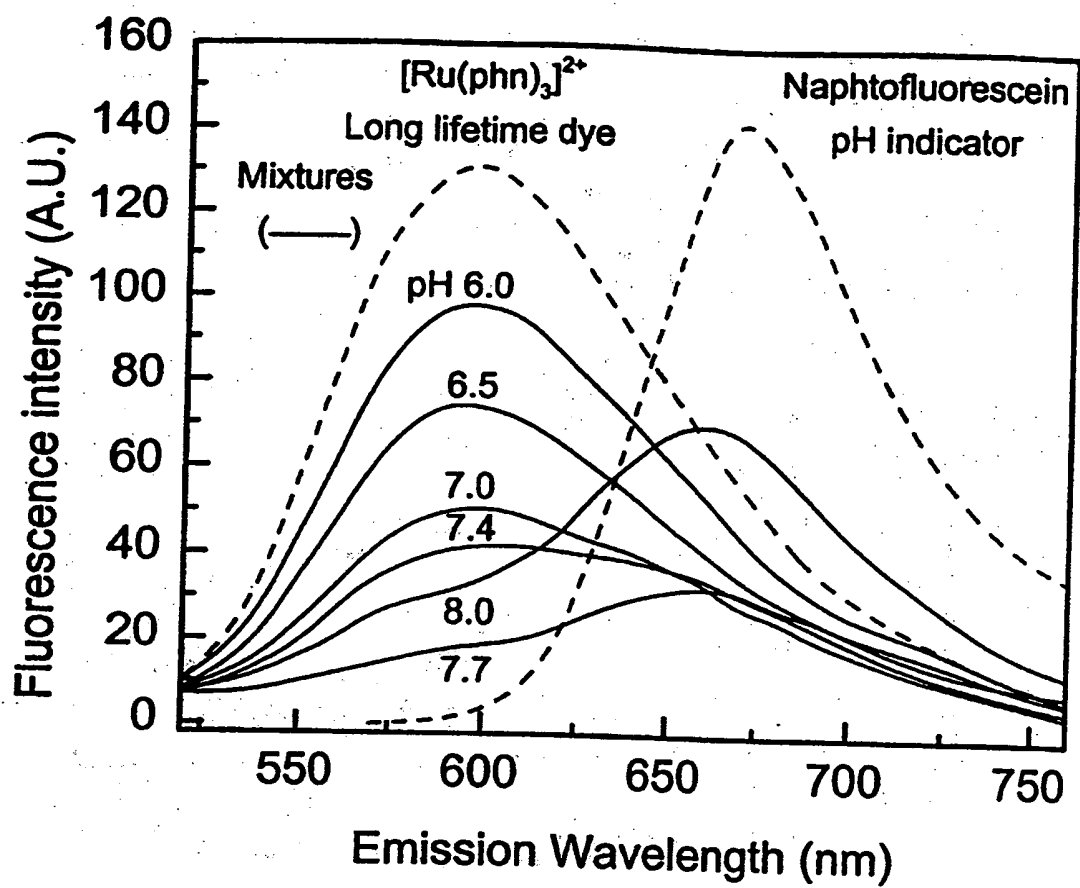
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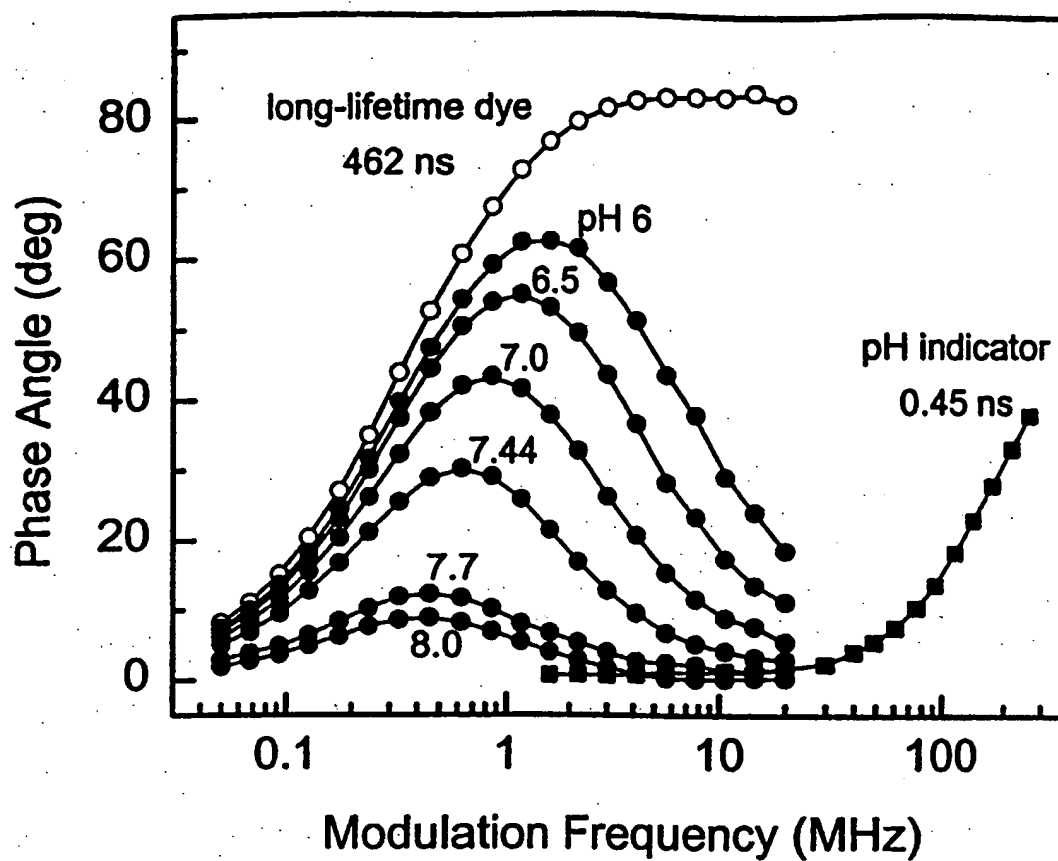
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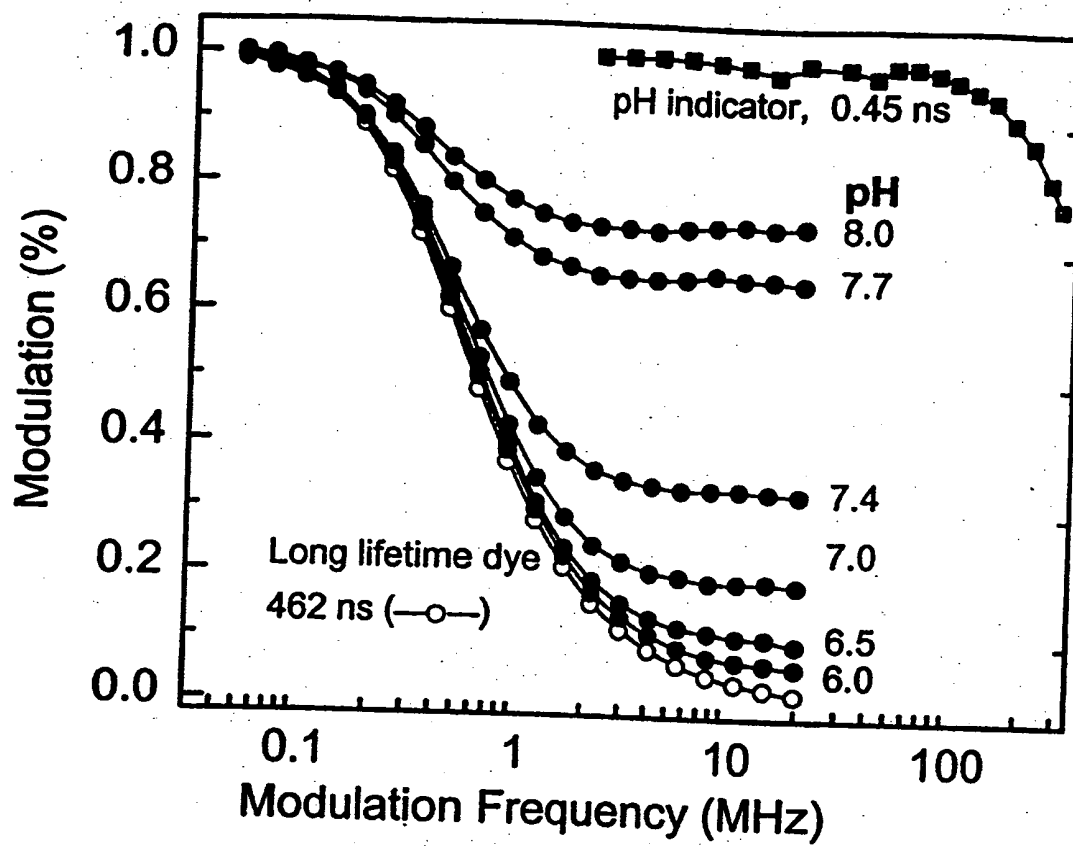
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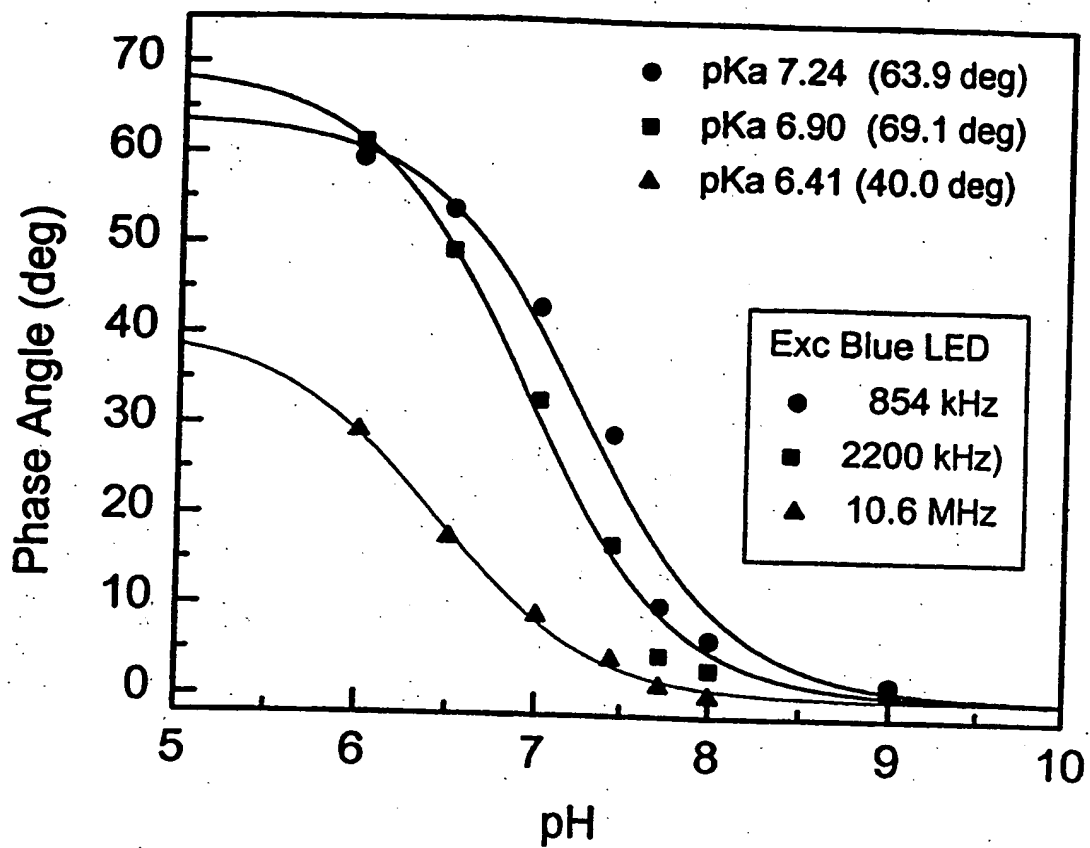
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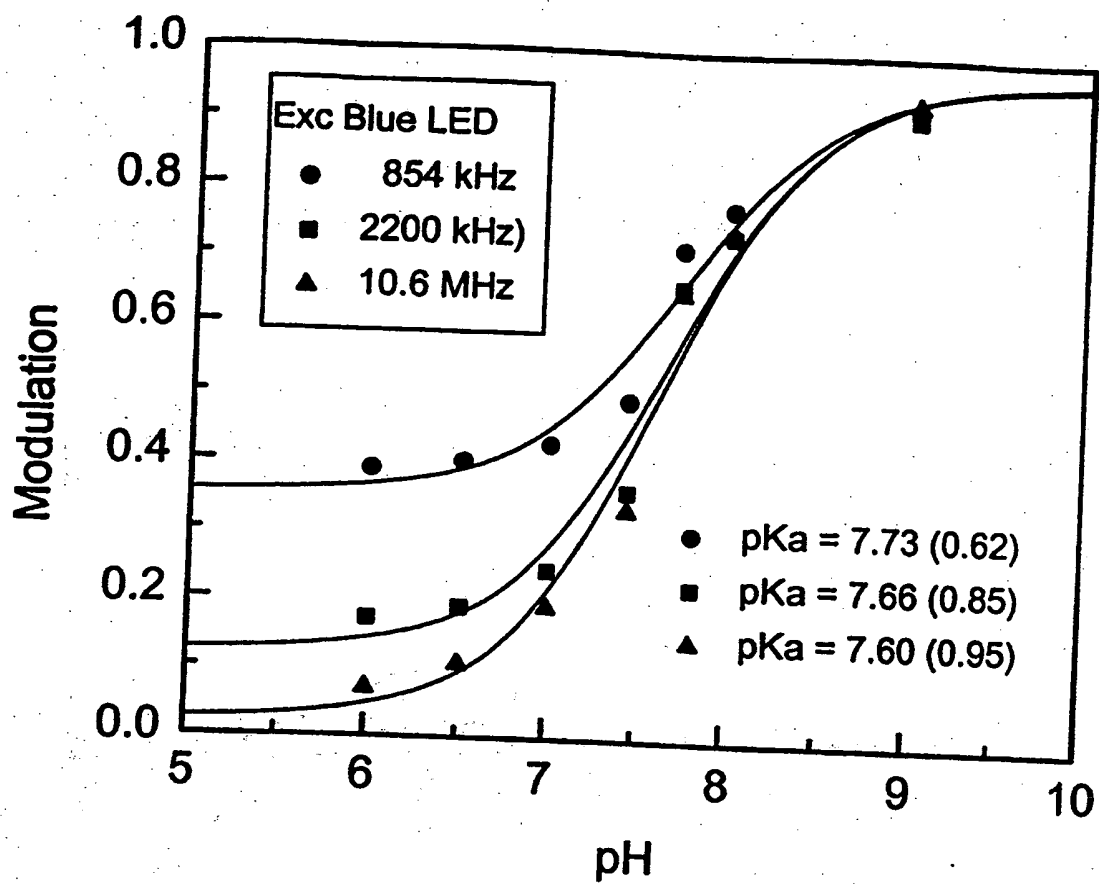
Fig. 15

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   : G0IN 21/64, 15/14

US CL   :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   250/461.2, 458.1, 461.1, 459.1, 461.2, 302; 436/172, 527, 521, 169, 501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, WPIDS, USPAT, DIALOG, MEDLINE

FLUORESC, PHASE, MODULATE, FREQUENT, PROBE, AMPLITUDE, LIFETIME, FLUORMET, POLYMERIC MATRI.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92/07245 A1 (THE UNIVERSITY OF MARYLAND) 30 April 1992, pages 3-43.	1-8
Y	WO 92/13265 A1 (THE UNIVERSITY OF MARYLAND) 06 August 1992, whole document, especially pages 9-48.	1-8
Y	US 5,270,548 A (STEINKAMP) 14 December 1993, whole document especially columns 3-10.	1-7
X	US 5,315,122 A (PINSKY et al) 24 May 1994, column 2, lines 59-68.	1-7

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 AUGUST 1999	**30 AUG 1999**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	PADMA BASKAR
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Form PCT/ISA/210 (second sheet)(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

250/461.2, 458.1, 461.1, 459.1, 461.2, 302; 436/172, 527, 521, 169, 501